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Thermodynamic Evidence for Conformational Coupling between the B and C Domains of the Mannitol Transporter of *Escherichia coli*, Enzyme II^{mtl}*

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The transport across the cytoplasmic membrane and concomitant phosphorylation of mannitol in *Escherichia coli* is catalyzed by the mannitol-specific transport protein from the phosphoenolpyruvate-dependent phosphotransferase system, enzyme II^{mtl}. Interactions between the cytoplasmic B and the membrane embedded C domain play an important role in the catalytic cycle of this enzyme, but the nature of this interaction is largely unknown. We have studied the thermodynamics of binding of (i) mannitol to enzyme II^{mtl}, (ii) the substrate analog perseitol to enzyme II^{mtl}, (iii) perseitol to phosphorylated enzyme II^{mtl}, and (iv) mannitol to enzyme II^{mtl} treated with trypsin to eliminate the cytoplasmic domains. Analysis of the heat capacity increment of these reactions showed that approximately 50–60 residues are involved in the binding of mannitol and perseitol, but far less in the phosphorylated state or after removal of the B domain. A model is proposed in which binding of mannitol leads to the formation of a contact interface between the two domains, either by folding of unstructured parts or by docking of preexisting surfaces, thus positioning the incoming mannitol close to the phosphorylation site on the B domain to facilitate the transfer of the phosphoryl group.

The transport of carbohydrates from the environment into the bacterial cell is, in many cases, accomplished by a complex of proteins that together make up the phosphoenolpyruvate-dependent phosphotransferase system (PTS)[1] (1, 2). This system consists of a number of transport proteins, each one specific for one carbohydrate, and a chain of cytoplasmic proteins that ultimately transfer a phosphoryl group derived from PEP to the incoming carbohydrate. The first protein in this chain is enzyme I, which accepts the phosphoryl group from PEP and transfers it to HPr, which in its phosphorylated form is the substrate for the transport proteins. All transport proteins of the PTS have a similar architecture, consisting of a part that binds and transports the carbohydrate and is embedded in the cytoplasmic membrane and two cytoplasmic parts, responsible for the phosphorylation of the substrate. The parts may or may not be covalently bound, and all possible combinations do, in fact, exist. In the case of the mannitol-specific transporter from *Escherichia coli*, termed enzyme II^{mannitol}, all three parts are covalently linked and are, thus, domains of the same protein. The phosphoryl group is accepted from P-HPr by His-554 in the C-terminal A domain and transferred to Cys-384 on the B domain. Mannitol is bound at the periplasmic side and translocated across the membrane by the N-terminal C domain, after which the carbohydrate is phosphorylated by the B domain. The protein is believed to function as a dimer, both in the membrane and solubilized in detergent (3–9).

It is obvious from the above that domain interactions play an important role in the catalytic cycle of this enzyme. Lolkema *et al.* (10), for instance, have shown that phosphorylation of the B domain causes a 1000-fold increase in the rate of isomerization of the mannitol-occupied C domain, the principle event in the transport process. Further evidence that the B and C domains influence each other's conformation was obtained from an analysis of the binding kinetics of mannitol to the wild-type enzyme, the subcloned C domain, and a number of mutants substituted at the position of the second phosphorylation site (10, 11). However, in the absence of knowledge of the three-dimensional structure in the intact complex, the nature of the interaction is still largely unknown. Thermodynamic data can give additional information, both on the nature of the interaction and also on the extent of the structural changes that are involved. We have chosen isothermal titration calorimetry (ITC) in this study to develop the required thermodynamic data.

In this study, we determine the energetics of mannitol binding to EII^{mtl} by ITC in inside-out membrane vesicles, *i.e.* under conditions that closely resemble the native state of the protein. To study the effect of phosphorylation, the binding of a substrate analog, perseitol, which cannot be transported or phosphorylated (12), to EII^{mtl} in the phosphorylated and unphosphorylated state is determined and compared with binding of mannitol. Finally, the effect of removal of the A and B domains on the functioning of the C domain is investigated. In all of these cases, the experiments have been performed at various temperatures, enabling us to calculate $\Delta C_p^o{}_{obs}$. This parameter has been shown to correlate with the change in solvent-exposed groups before and after the binding reaction (see, *e.g.*, Refs. 13–15) and can, therefore, be used to monitor structural rearrangements taking place in the enzyme upon binding of the substrate.

EXPERIMENTAL PROCEDURES

Materials—D-[1-^{14}C]Mannitol (2.04 GBq/mmol) was obtained from Amersham Pharmacia Biotech; D-[1-^{3}H]mannitol (976.8 GBq/mmol) was from NEN Life Science Products. Perseitol was obtained from Pfanstiehl Laboratories Inc., Waukagan, IL. TPCK-treated trypsin was from Worthington or Sigma, and bovine pancreatic trypsin inhibitor

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[1] The abbreviations used are: PTS, phosphoenolpyruvate-dependent phosphotransferase system; DTT, dithiothreitol; EII^{mtl}, enzyme II^{mtl}; HA, high affinity; HPr, histidine-containing protein; LA, low affinity; mtl, mannitol; PEP, phosphoenolpyruvate; ITC, isothermal titration calorimetry; TPCK, tosylphenylalanyl chloromethyl ketone.

was from Sigma. Enzyme I and HPr were purified as described previously (16, 17). Decylpoly(ethylene glycol) 300 was synthesized by B. Kwant at the Department of Chemistry, University of Groningen. All other chemicals were of the highest purity available.

Growth of Bacteria and Preparation of Membrane Vesicles—Membrane vesicles containing large amounts of EII^{mtl} were obtained from *E. coli* strain LGS322 (F$^-$ *thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gatR49, gatA50, Δ(mtlA'p), mtlD^c, D(gutR'M-DBA-recA)*), which contains a chromosomal deletion for the wild type *mtlA* gene, transformed with the inducible plasmid pMamtlA (18), encoding for EII^{mtl}. A single colony was used to inoculate 10 ml of LB medium (10 g/liter Bactotryptone, 5 g/liter Bactoyeast extract, 10 g/liter NaCl) containing 100 μg/ml ampicillin. This culture was grown at 30 °C to an A^{600} of approximately 0.6 and then used to inoculate a 1.5-liter culture in the same medium, which was grown overnight to an A^{600} of approximately 3. This was used to inoculate a 60-liter fermenter containing the same medium. The cells were grown to an A^{600} of 0.7, induced for 2 h at a temperature of 42 °C and harvested. The final A^{600} was 0.85, resulting in a total of 86 g of cells (wet weight). The cells were washed three times with ~2 liters of buffer (50 mM NaP_i, pH 7.5, 1 mM NaN_3) and stored on ice overnight. From this preparation, inside-out membrane vesicles were obtained as described by Lolkema and Robillard (8). The final yield was 100 ml of vesicle solution containing 30 μM EII^{mtl}. The preparation was stored in 2-ml aliquots at −80 °C until use. Approximately 30% of the total protein was EII^{mtl}, analyzed by SDS-polyacrylamide gel electrophoresis. The orientation of the protein with respect to the membrane was determined from the mannitol phosphorylation activity before and after treatment with trypsin (5) and turned out to be approximately 90% inside-out.

Membrane vesicles not containing EII^{mtl} were prepared from *E. coli* LGS322 without the plasmid. In this case, 9 liters of LB medium (without ampicillin) was inoculated 1:50 and grown to an A^{600} of 0.9 and then induced in the manner described above. Membrane vesicles were prepared as described for the cells containing the plasmid.

PEP-dependent Mannitol Phosphorylation—The PEP-dependent phosphorylation of mannitol by EII^{mtl} was determined as described (19).

Concentration Determination—The concentration of EII^{mtl} in the vesicles was determined by the mannitol burst method, which quantitates the amount of phosphorylation sites present in the preparation (20). 20 nM enzyme I and 1 μM HPr were added to three aliquots of 160 μl of a standard buffer solution (25 mM Tris-HCl, pH 8.3, 5 mM DTT, 5 mM $MgCl_2$, 5 mM PEP, 7 mM decylmaltoside). Under these conditions, the kinetics of conversion of mannitol to mannitol-1-P are slow and limited by the transfer of the phosphoryl group from P-HPr to the A domain of EII^{mtl}. A 10 mM [^{3}H]mannitol solution (20 μl) was added to one aliquot. In this case, the mannitol phosphorylation reaction was started by adding 40 μl of the vesicle solution and monitored by applying 20-μl aliquots to a small Dowex column at 20-s intervals as described (19). A vesicle solution (40 μl) was added to each of the other two aliquots and incubated at 30 °C, one for 10 and the other for 30 min to allow for complete phosphorylation of EII^{mtl}. The assay was then started by adding 20 μl of the mannitol solution to each mixture and monitored in the same way. No differences were observed between the samples incubated for 10 and 30 min in the absence of mannitol, indicating that phosphorylation of EII^{mtl} is completed within 10 min. The change in the concentration of mannitol-1-P in time was extrapolated back to $t = 0$ to determine the difference in the mannitol-1-P concentration between samples incubated with and without PEP. This difference equals the concentration phosphorylation sites on EII^{mtl} in the preparation, assuming that the transfer of all the EII^{mtl} phosphoryl to mannitol is rapid and complete. Since EII^{mtl} contains two phosphorylation sites per monomer, this number is divided by two to obtain the concentration EII^{mtl}.

Isothermal Titration Calorimetry—All titrations were performed using a MCS isothermal titration calorimeter from Microcal. A similar instrument has been described elsewhere (21). During experiments, the vesicle solution was thermostatted at the desired temperature and stirred at 700 rpm. The injection sequence consisted of 30 injections of 3 μl each from a 100-μl syringe, unless indicated otherwise. Data were analyzed using Origin software from Microcal.

Samples containing vesicles were dialyzed overnight against 1 liter of buffer (50 mM KP_i or Tris/HCl, pH 7.5, 2 mM EDTA, 1 mM DTT) and, if necessary, diluted in the dialysis buffer before the experiment. Mannitol and perseitol solutions were prepared from stock solutions in H_2O (30.69 and 13.64 mM, respectively) by dilution in dialysis buffer to minimize artifacts arising from mixing the two different buffer solutions during the ITC experiment. Control experiments in which the mannitol or perseitol solution was injected into the buffer were routinely run and always showed negligible peaks. All solutions were degassed by gently stirring under vacuum directly before measurements.

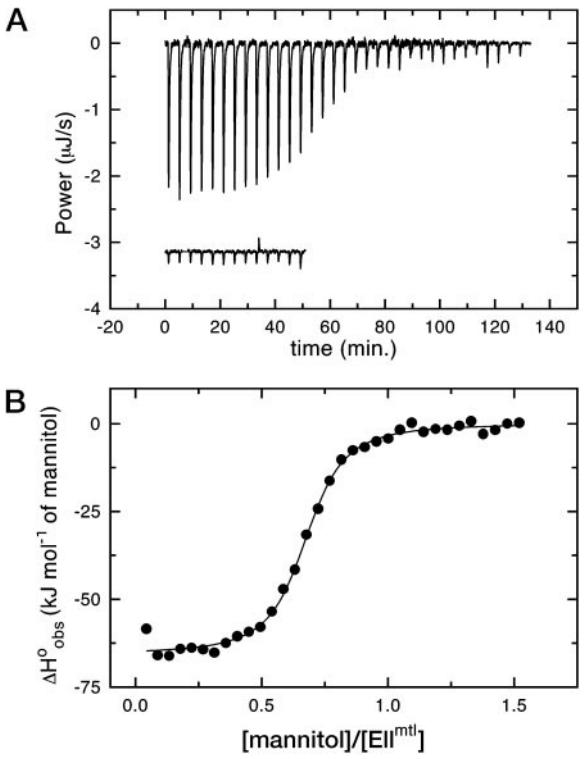

FIG. 1. **Raw titration calorimetry data.** *A*, 33 injections of 3 μl each of a 300 μM mannitol solution into a solution of 15 μM EII^{mtl} in inside-out membrane vesicles in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 1 mM DTT at 20 °C (*upper trace*). For comparison, 13 injections of the same mannitol solution into a solution of membrane vesicles without EII^{mtl} are also shown (*lower trace*). The peaks result from mixing of the two solutions. *B*, integrated heats of injection for the experiment shown in the *top panel*. The *solid line* represents the best fit of the one-set-of-sites model to the data. The parameters derived from the fit are: $n = 0.67 \pm 0.01$, $K_d = 115 \pm 17$ nM, and $\Delta H^o_{obs} = -63.4 \pm 0.9$ kJ mol^{-1}.

To study the interaction of perseitol with phosphorylated EII^{mtl}, the procedure was slightly modified. After dialysis overnight, 200 ml of the dialysis buffer was taken and brought to 7 mM in $MgCl_2$ and 2 mM in PEP. At the same time, small volumes of a 80 μM HPr and 100 mM PEP solution were added to 2 or 4 ml of the vesicles solution to give final concentrations of 5.5 μM and 2 mM, respectively. The vesicle solution was then reequilibrated with the modified buffer by dialysis for 4 h. Before calorimetric experiments, a small volume of a 3.3 μM enzyme I solution was added to the vesicles solution to yield a final enzyme I concentration of 120 nM. The solution was incubated at room temperature for 10 min before loading the sample into the calorimeter. Because of the low enthalpy of binding in this reaction, the injection sequence was changed to 16 injections of 6 μl each.

Vesicles containing only the C domain in the inside-out orientation were prepared from EII^{mtl}-containing vesicles by proteolysis, using 10 μg/ml TPCK-treated trypsin for 45 min at room temperature. To stop the reaction, soybean trypsin inhibitor was added to a final concentration of 100 μg/ml, after which the normal dialysis procedure was followed. The success of the treatment was evaluated by monitoring mannitol phosphorylation activity and by SDS-polyacrylamide gel electrophoresis. Intact EII^{mtl} runs at an apparent molecular mass of 56 kDa and the C domain at 30 kDa.

RESULTS

Binding of Mannitol to EII^{mtl}—Fig. 1*A* shows a typical example of the raw data obtained from a titration of EII^{mtl}-containing inside-out vesicles with mannitol. Upon addition of small aliquots of the mannitol solution, exothermic signals are observed that, after saturation of the binding, become small and constant. For comparison, a titration of a solution of a

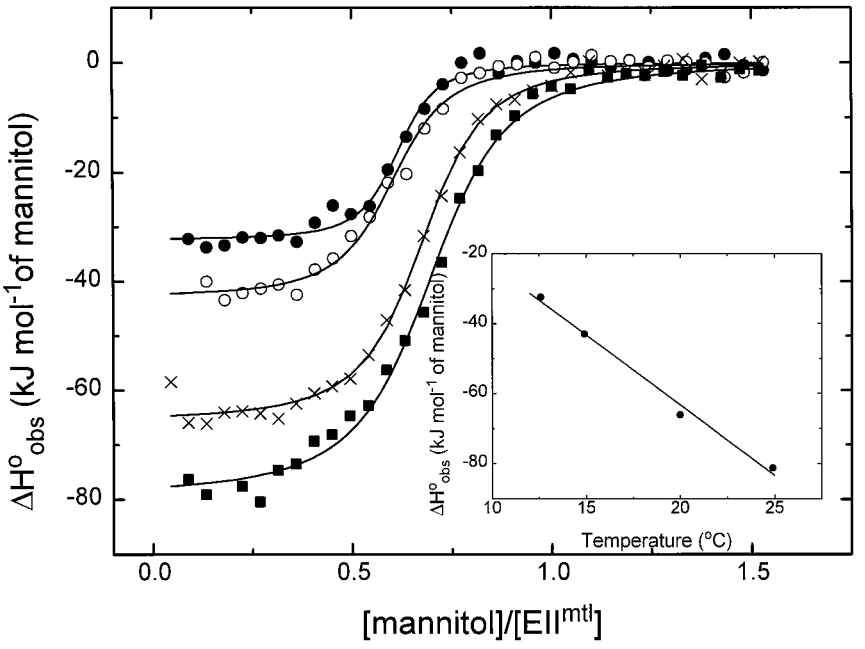

FIG. 2. **Integrated heats of injection for the titration of EII^{mtl} with mannitol in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 1 mM DTT at 12.6 °C (●), 14.9 °C (○), 20.0 °C (×), and 24.9 °C (■).** The *solid lines* represent the best fit of the one-set-of-sites model to the data. *Inset*, the variation of ΔH^o_{obs} with temperature. The *solid line* is obtained from linear least squares analysis and has a slope of -4.0 ± 0.3 kJ K^{-1} mol^{-1}.

TABLE I
Mannitol binding by EII^{mtl}

Parameters are derived from a nonlinear least squares fit to the data, using the one-set-of-sites model. Errors are the standard deviations obtained from the fits.

[EII^{mtl}]	[mtl]	T	n	K_d	ΔH^o_{obs}	ΔG^o_{obs}	$-T\,\Delta S^o_{obs}$
μM	μM	°C		nM		kJ mol^{-1} of mannitol	
14.9[a]	300	12.6	0.60 ± 0.01	61 ± 14	−32.4 ± 0.6	−39.5 ± 0.6	−7.1 ± 0.9
14.9[a]	300	14.9	0.59 ± 0.01	131 ± 23	−42.9 ± 0.9	−37.9 ± 0.5	5.0 ± 1.0
15.0[a,d]	300	20.0	0.67 ± 0.01	141 ± 25	−66.1 ± 2.4	−38.5 ± 0.4	27.6 ± 2.7
15.0[a]	300	24.9	0.76 ± .001	223 ± 26	−81.2 ± 1.2	−38.0 ± 0.3	43.2 ± 1.3
15.0[b]	300	14.9	0.63 ± 0.01	65 ± 10	−31.3 ± 0.5	−39.7 ± 0.4	−8.4 ± 0.7
14.9[b]	300	20.7	0.68 ± 0.01	88 ± 11	−47.7 ± 0.7	−39.7 ± 0.3	8.0 ± 0.8
14.9[b]	301	25.2	0.65 ± 0.01	89 ± 10	−66.5 ± 0.9	−40.3 ± 0.3	26.2 ± 1.0
20.0[c]	200	20.4	0.46 ± 0.01	260 ± 26	−62.8 ± 0.8	−37.0 ± 0.3	25.8 ± 0.9

[a] In 50 mM KP_i, pH 7.5, 2 mM EDTA, 1 mM DTT.
[b] In 50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 mM DTT.
[c] In 50 mM Hepes/NaOH, pH 7.5, 2 mM EDTA, 1 mM DTT.
[d] Average of three separate experiments; errors are the standard deviations of the mean.

similar concentration of vesicles prepared from *E. coli* LGS322 cells without the plasmid encoding for EII^{mtl} is also shown. The resulting signals are small and constant, indicating the lack of specific binding of mannitol in this case. We can, therefore, conclude that the observed effect is due to the specific binding of mannitol to EII^{mtl}.

The integrated heats for each injection were calculated from the areas under the peaks and, after correction for the heat of dilution of the mannitol solution, fitted to a mathematical binding model assuming one set of sites (Fig. 1*B*). The model uses three parameters: the stoichiometry of the reaction n, the association constant K_a, and the enthalpy change associated with binding ΔH^o_{obs} (21). The heat resulting from the first injection often was somewhat smaller than heats generated from subsequent injections, probably due to imperfect filling or dilution of the mannitol solution at the tip of the syringe during equilibration of the calorimeter, and was, therefore, not taken into account during data analysis. The model fits the data well and yields an estimate of -63.4 ± 0.9 kJ mol^{-1} for ΔH^o_{obs}, 115 ± 17 nM for the dissociation constant K_d, and 0.67 ± 0.01 for the stoichiometry of the reaction at 20 °C under the conditions given in the figure legend.

The heat capacity increment of the binding reaction, $\Delta C_p{}^o_{obs}$, can be obtained from the temperature dependence of the enthalpy of binding. Fig. 2 shows the results of titrations of EII^{mtl} with mannitol at various temperatures. The calculated thermodynamic parameters obtained from these data are listed in Table I. The variation of ΔH^o_{obs} as a function of temperature is linear over the temperature interval that we have investigated (Fig. 2, *inset*), resulting in a value of -4.0 ± 0.3 kJ K^{-1} mol^{-1} for $\Delta C_p{}^o_{obs}$ in phosphate buffer.

In general, there are two terms that contribute to ΔH^o_{obs}: a term arising directly from the binding reaction and a term rising from a change in protonation state of the protein in the complexed and uncomplexed state.

$$\Delta H^o_{obs} = \Delta H^o_0 + N_{H+}\,\Delta H^b_{ion} \qquad \text{(Eq. 1)}$$

N_{H+} is the number of protons released by the buffer and ΔH^b_{ion} is the ionization enthalpy of the buffer (5.3 kJ mol^{-1} for phosphate buffer; Ref. 22). To determine whether protons are released or taken up by the buffer as a result of the binding process, the experiment was repeated in 50 mM Hepes and Tris buffers at pH 7.5 (ΔH^b_{ion} 21 and 47.5 kJ mol^{-1}, respectively). The binding of mannitol becomes less exothermic when ΔH^b_{ion} increases (Table I), indicating that protons are released to the buffer, with a calculated value for N_{H+}/mol of mannitol of 0.45 ± 0.1 at 20 °C. The $\Delta C_p{}^o_{obs}$ for the interaction of EII^{mtl}

with mannitol in Tris buffer is -3.4 ± 0.4 kJ K^{-1} mol^{-1}.

The Binding of Perseitol to EII^{mtl}—Perseitol is a structural analogue of mannitol (Fig. 3) that binds to EII^{mtl}, but is not phosphorylated or transported across the membrane (12, 23). It inhibits the transport activity of the enzyme competitively, indicating that the binding sites for mannitol and perseitol are the same. The results of ITC experiments in which EII^{mtl} is titrated with perseitol at temperatures between 10.5 and 30 °C are presented in Fig. 4 and Table II. The binding of perseitol can also be described by a one-set-of-sites model, using parameters that differ very little from the parameters that were obtained for the binding of mannitol to EII^{mtl}. The apparent K_d for perseitol binding is larger by a factor of 1.5–2 compared with mannitol, whereas the ΔH of binding is about 5 kJ mol^{-1} less exothermic over the entire temperature interval. $\Delta C_p{}^o{}_{obs}$ for binding of perseitol is -3.9 ± 0.2 kJ K^{-1} mol^{-1}, very close to the value for mannitol binding under the same conditions. It, therefore, seems that perseitol is a good model compound to study the changes in binding affinity upon phosphorylation of EII^{mtl}, since the protein can not be dephosphorylated by perseitol.

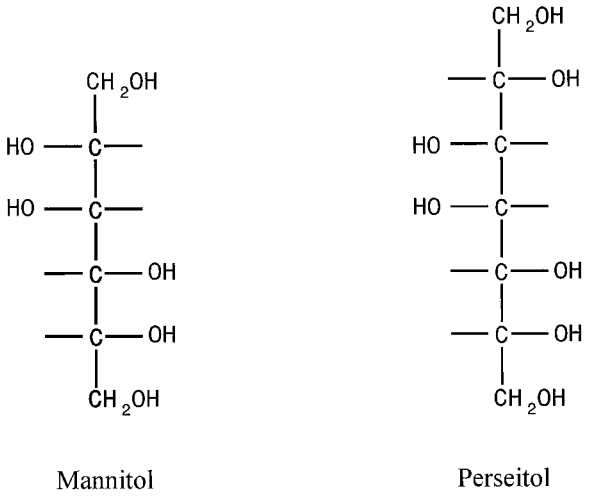

FIG. 3. **Mannitol (*left*) and perseitol (*right*).** Both molecules are shown in Fisher projection.

The Binding of Perseitol to Phosphorylated EII^{mtl}—The binding of perseitol to the phosphorylated state of EII^{mtl} proved to be difficult to follow by ITC because we observed only a small enthalpy change associated with the reaction, resulting in a low signal to noise ratio. In addition, measurements at temperatures above 25 °C were hampered by instability of the base line, possibly due to aggregation during the experiments. Nonetheless, we were able to determine the binding parameters for the interaction at two different temperatures, allowing us to roughly estimate $\Delta C_p{}^o{}_{obs}$ (Fig. 5 and Table III). Phosphorylated EII^{mtl} binds perseitol with an affinity comparable to the unphosphorylated enzyme, but the contributions of $\Delta H^o{}_{obs}$ and $\Delta S^o{}_{obs}$ to the free energy of binding differ considerably in both cases. In the case of the non-phosphorylated enzyme, there is a much larger contribution of the enthalpy of binding to the stabilization of the complex than when the enzyme is in its phosphorylated state. This is compensated for, however, by a larger contribution of the entropy term, an effect that has been termed enthalpy-entropy compensation and has been observed for a large number of non-covalent interactions (see "Discus-

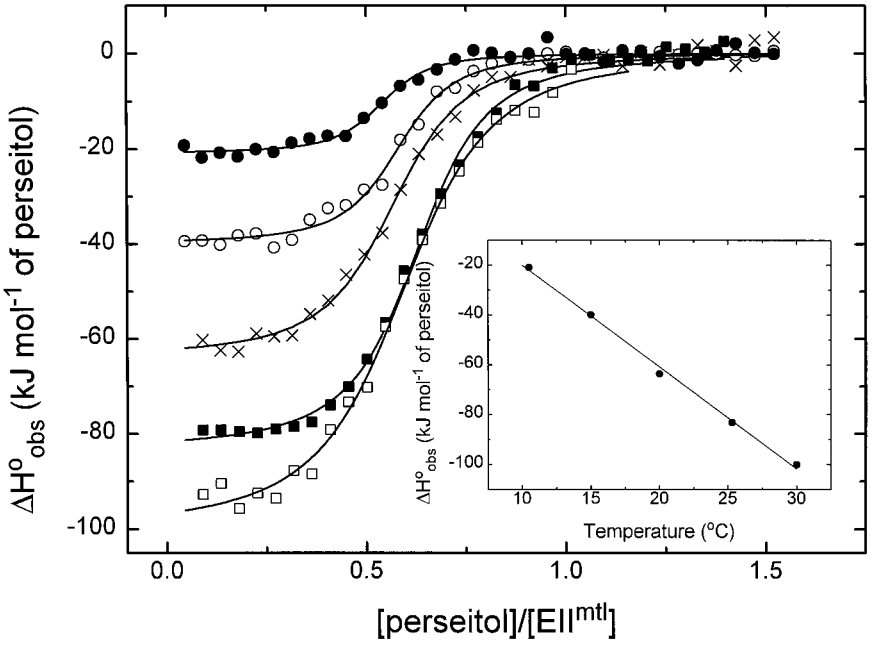

FIG. 4. **Integrated heats of injection for the titration of EII^{mtl} with perseitol in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 1 mM DTT at 10.5 °C (●), 15.0 °C (○), 20.0 °C (×), 25.3 °C (■), and 30.0 °C (□).** The *solid lines* represent the best fit of the one-set-of-sites model to the data. *Inset*, the variation of $\Delta H^o{}_{obs}$ with temperature. The *solid line* is obtained from linear least squares analysis and has a slope of -3.9 ± 0.2 kJ K^{-1} mol^{-1}.

TABLE II
Perseitol binding by EII^{mtl}

Parameters are derived from a nonlinear least squares fit to the data, using the one-set-of-sites model. Errors are the standard deviations obtained from the fits. All data were recorded in 50 mM KP$_i$, pH 7.5, 2 mM EDTA, 1 mM DTT.

[EII^{mtl}]	[ptl]	T	n	K_d	$\Delta H^o{}_{obs}$	$\Delta G^o{}_{obs}$	$-T\,\Delta S^o{}_{obs}$
μM	μM	°C		nM	*kJ mol^{-1} of perseitol*		
15.0	300	10.5	0.52 ± 0.01	109 ± 30	−20.9 ± 0.6	−37.8 ± 0.7	−16.9 ± 0.9
15.0	300	15.0	0.57 ± 0.01	141 ± 20	−39.9 ± 0.6	−37.8 ± 0.4	2.1 ± 0.8
15.0	300	20.0	0.56 ± 0.01	224 ± 27	−63.6 ± 1.0	−37.3 ± 0.3	26.3 ± 1.1
14.8	300	25.3	0.62 ± 0.01	199 ± 19	−83.2 ± 1.0	−38.3 ± 0.3	44.9 ± 1.1
14.8	300	30.0	0.59 ± 0.01	323 ± 30	−100.0 ± 1.4	−37.6 ± 0.3	62.4 ± 1.5

sion"). $\Delta C_p{}^o{}_{obs}$ of the binding reaction of perseitol with phosphorylated EII^{mtl} is less than 1 kJ K^{-1} mol^{-1}, both in phosphate and Tris buffers, *i.e.* less than 25% of the value for the non-phosphorylated enzyme.

The Binding of Mannitol to Trypsin-treated EII^{mtl}—Mild trypsinolysis of EII^{mtl}-containing membrane vesicles leads to the formation of two fragments of approximately equal molecular weight (5), an N-terminal fragment, the C domain of EII^{mtl}, responsible for mannitol binding and transport across the membrane, and a C-terminal fragment consisting of the cytoplasmic A and B domains. At longer exposure times, trypsin fully degrades the A and B domains, but the C domain is protected from degradation, apparently as a result of its membrane environment. Removal of the A and B domains leaves the structural integrity and transport capability of the C domain intact, since upon complementation with a purified combination of the B and A domains, IIBA^{mtl}, the activity of the system is restored (24). We have used this approach here to eliminate the A and B domains of EII^{mtl}, enabling us to study the influence of domain interactions on mannitol binding. The results are shown in Fig. 6 and Table IV. Removal of the A and B domains results in an increase in K_d to values ranging from 200 to 400 nM, depending on temperature (Table IV). $\Delta H^o{}_{obs}$ is around 57 kJ mol^{-1} and does not vary much with temperature compared with the wild type enzyme. The resulting value for $\Delta C_p{}^o{}_{obs}$ is -0.5 ± 0.2 kJ K^{-1} mol^{-1}, much smaller than in the in the intact enzyme with the A and B domains present. All values determined for $\Delta C_p{}^o{}_{obs}$ in this study are listed in Table V.

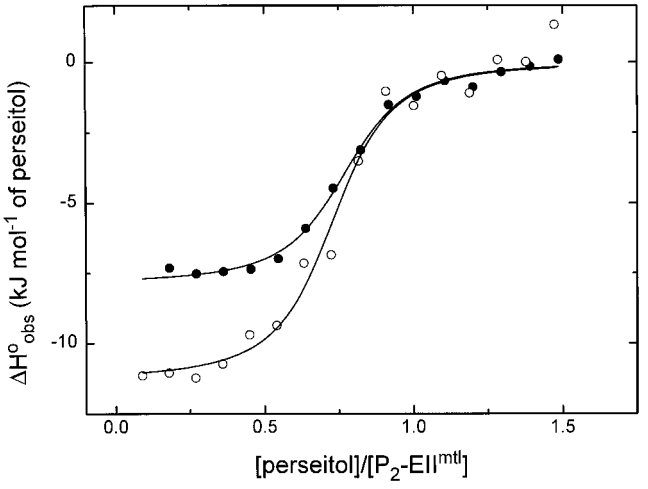

FIG. 5. **Integrated heats of injection for the titration of phosphorylated EII^{mtl} with perseitol in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 1 mM DTT at 20.5 °C (●) and 25.3 °C (○).** The *solid lines* represent the best fit of the one-set-of-sites model to the data.

DISCUSSION

Validity of the One-set-of-sites Model for Binding of Mannitol to EII^{mtl}—To be able to quantitatively interpret the data obtained from isothermal titration calorimetry experiments, one must choose a mathematical model to analyze the data. Previ-

TABLE III
Perseitol binding by phosphorylated EII^{mtl}

Parameters are derived from a nonlinear least squares fit to the data, using the one-set-of-sites model. Errors are the standard deviations obtained from the fits. Phosphorylation was achieved *in situ* by the presence of 5 μM HPr, 120 nM enzyme I, and 2 mM PEP as described under "Experimental Procedures."

[EII^{mtl}]	[ptl]	T	n	K_d	$\Delta H^o{}_{obs}$	$\Delta G^o{}_{obs}$	$-T\,\Delta S^o{}_{obs}$
μM	μM	°C		nM	*kJ mol^{-1} of perseitol*		
26.6[a]	534	20.5	0.74 ± 0.01	410 ± 66	−7.9 ± 0.2	−35.9 ± 0.4	−28.0 ± 0.5
12.9[a]	258	25.3	0.70 ± 0.02	184 ± 64	−11.3 ± 0.5	−38.5 ± 0.9	−27.2 ± 1.1
15.0[b]	300	21.5	0.67 ± 0.02	493 ± 119	−16.6 ± 0.7	−35.6 ± 0.6	−19.0 ± 1.0
14.0[b]	300	25.2	0.66 ± 0.02	550 ± 106	−17.0 ± 0.7	−35.8 ± 0.5	−18.8 ± 0.9

[a] In 50 mM KP_i, pH 7.5, 2 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$, 2 mM PEP.
[b] In 50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 7 mM $MgCl_2$, 2 mM PEP.

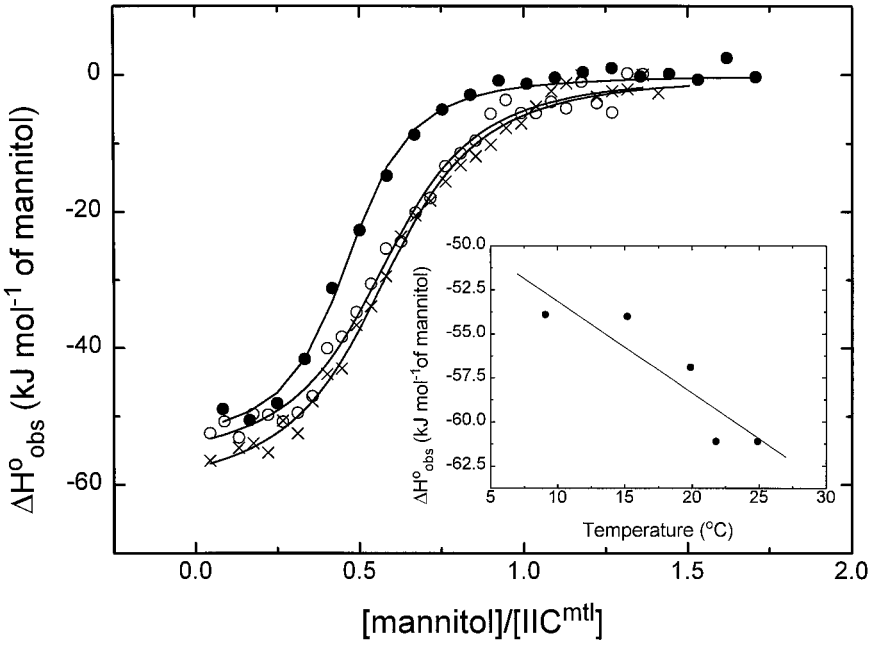

FIG. 6. **Integrated heats of injection for the titration of C domain of enzyme II^{mtl} obtained from trypsinolysis (*IIC^{mtl}*) with mannitol in 50 mM potassium phosphate, pH 7.5, 2 mM EDTA, 1 mM DTT at 9.1 °C (●), 19.9 °C (○), and 24.9 °C (×).** For clarity, not all data are shown. The *solid lines* represent the best fit of the one-set-of-sites model to the data. *Inset*, the variation of $\Delta H^o{}_{obs}$ with temperature. The *solid line* is obtained from linear least squares analysis and has a slope of -0.5 ± 0.2 kJ K^{-1} mol^{-1}.

TABLE IV
Mannitol binding by IIC^{mtl}, prepared from EII^{mtl} by trypsinolysis

Parameters are derived from a non-linear least squares fit to the data, using the one-set-of-sites-model. Errors are the standard deviations obtained from the fits.

[IIC^{mtl}]	[mtl]	T	n	K_d	ΔH^o_{obs}	ΔG^o_{obs}	$-T\,\Delta S^o_{obs}$
μM	μM	°C		nM		kJ mol^{-1} of mannitol	
13.5[a]	300	9.1	0.45 ± 0.01	303 ± 46	−53.9 ± 1.3	−35.2 ± 0.4	18.7 ± 1.4
10.1[a]	200	15.2	0.52 ± 0.01	224 ± 17	−54.0 ± 0.7	−36.7 ± 0.2	17.3 ± 0.8
10.1[a]	200	19.9	0.59 ± 0.01	392 ± 46	−56.9 ± 1.2	−35.9 ± 0.3	21.0 ± 1.3
5.9[a]	120	21.8	0.4 ± 0.01	150 ± 27	−61.1 ± 2.2	−38.5 ± 0.5	22.6 ± 0.3
10.1[a]	200	24.9	0.59 ± 0.01	413 ± 39	−61.1 ± 1.1	−36.4 ± 0.3	24.7 ± 1.2
13.3[b]	150	15.2	0.55 ± 0.01	265 ± 32	−58.4 ± 1.1	−36.3 ± 0.3	22.1 ± 1.2

[a] In 50 mM KP_i, pH 7.5, 2 mM EDTA, 1 mM DTT.
[b] In 50 mM Tris/HCl, pH 7.5, 2 mM EDTA, 1 mM DTT.

TABLE V
The observed heat capacity increment, $\Delta C_p{}^o{}_{obs}$, and the theoretical number of residues involved in substrate binding, R^{th}

$\Delta C_p{}^o{}_{obs}$ was calculated from the data listed in Tables I–IV; R^{th} was calculated as described (15).

Reaction	Buffer	$\Delta C_p{}^o{}_{obs}$	R^{th}
		kJ K^{-1} mol^{-1} of ligand	
EII^{mtl} + mannitol	Phosphate	−4.0 ± 0.3	60
EII^{mtl} + mannitol	Tris	−3.4 ± 0.4	47
EII^{mtl} + perseitol	Phosphate	−3.9 ± 0.2	60
P_2-EII^{mtl} + perseitol	Phosphate, Tris	<1	ND
IIC^{mtl} + mannitol	Phosphate	−0.5 ± 0.2	<4

ous binding studies on substrate binding by EII^{mtl} using equilibrium dialysis or filtration techniques indicated that there are two binding sites for mannitol on the EII^{mtl} dimer, a high affinity site (HA) with a K_d of approximately 45–600 nM and a low affinity site (LA) with K_d values of 5–10 μM (25–27). Binding studies using flow dialysis resulted in values ranging from 45 to 212 nM for the HA binding site (11, 12), whereas the LA site could not be assessed with this technique. Based on this information, a binding model describing two possible binding sites with different affinities would seem to be the appropriate choice. However, attempts to fit the ITC data with such a model did not give sensible results and were, therefore, not further pursued. An explanation for this apparent discrepancy may be found in the values of the parameter c, the quotient of the concentration of sites in the calorimetric cell and the $K_{d,c}$ should be between 1 and 1000 to be able to calculate the thermodynamic parameters reliably (21). Assuming a value of 5.2 μM for the K_d of the LA site (27), the value of the c parameter for this site is just above 1 in our experiments. For comparison, the values of c calculated from the data in Table I range from 32 to 210 and, therefore, it is not surprising that the observed dependence of the enthalpy on the molar ratio of mannitol and EII^{mtl} is totally dominated by binding to the HA site. Further support for our interpretation comes from the fact that the observed dissociation constants for the HA site in phosphate and Tris buffer are in good agreement with values that were measured previously in inside-out membrane vesicles containing EII^{mtl}, using other techniques.

The average value of the stoichiometry calculated from the data in Table I in the absence of detergent is 0.62 ± 0.06, somewhat higher than the expected value of 0.5 for one HA site per EII^{mtl} dimer. This is probably due to inaccuracies in the concentration determination of EII^{mtl}, possibly resulting from oxidation of a small fraction of the protein. The active site cysteine of the B domain, Cys-384, is the site most prone to oxidation, a process that results in the loss of phosphorylation activity but not substrate binding. Since our concentration determination is based on the former, this would result in an underestimation of the protein concentration and, therefore, an overestimation of the binding stoichiometry. It should be noted that introducing an erroneous concentration of binding sites into the data analysis does not alter the values obtained for the K_d and ΔH^o_{obs} of the reaction (28).

Enthalpy-Entropy Compensation—Although the range of reported enthalpies in the reactions investigated in this study is almost 80 kJ mol^{-1}, the value of ΔG^o_{obs} varies only over a very narrow range (~6 kJ mol^{-1}) for the entire data set. This phenomenon is termed enthalpy-entropy compensation and can be illustrated in a plot of ΔH^o_{obs} *versus* ΔS^o_{obs} (Fig. 7). In the case of complete compensation, the expected slope of such a plot is the experimental temperature (29). For our data at 25 °C, the temperature at which we have the most complete data set with the largest range of ΔH^o_{obs} and ΔS^o_{obs}, we find a slope of 303 ± 8 K, equal within error to the expected value of 298 K. This is additional evidence that the model we have chosen to represent our data is correct. It also implies that the temperature dependence of the enthalpy and entropy functions are dominated by solvent reorganization, or more specifically, by the removal of water from surfaces that become buried upon binding of the substrate.

Dependence of Ligand Binding on Temperature—The heat capacity increment of the binding reaction, $\Delta C_p{}^o{}_{obs}$, for the reaction of EII^{mtl} and mannitol is −4.0 ± 0.3 kJ K^{-1} mol^{-1} in phosphate buffer. Qualitatively, this estimate can be interpreted as a conformational change of the transporter, resulting in removal of hydrophobic groups from the surrounding water (30). For a more quantitative analysis, care should be taken that the determination of $\Delta C_p{}^o{}_{obs}$ is not hampered by protonation effects, which can have a large influence on ΔH^o_{obs} and $\Delta C_p{}^o{}_{obs}$ of binding (31). Simulations have shown that the largest deviations of $\Delta C_p{}^o{}_{obs}$ from the intrinsic value are expected at the pK_a values of the complex and of the free protein. They are, at constant pH, a smooth function of the ionization enthalpy of the buffer and change in sign when the ionization enthalpies of protein and buffer are approximately equal. In the case of EII^{mtl}, the most likely candidate to undergo a change in protonation state is a histidine (11), with an approximate ionization enthalpy of 30 kJ mol^{-1}. We have determined $\Delta C_p{}^o{}_{obs}$ in phosphate and Tris buffers with ionization enthalpies that bracket the ionization enthalpy of histidine, and found that they differ by no more than 18% of the lowest value between the two. Therefore we conclude that the values we have determined are close to the intrinsic value for the $\Delta C_p{}^o{}_{obs}$ of the binding reaction of mannitol and EII^{mtl}.

The values for $\Delta C_p{}^o{}_{obs}$ for the binding of mannitol and perseitol to EII^{mtl} are large when compared with other binding processes. For example, the $\Delta C_p{}^o{}_{obs}$ for binding of serine to the serine receptor of bacterial chemotaxis is approximately −0.7 kJ K^{-1} mol^{-1} (32) and for binding of glucose to yeast hexokinase a value of −0.2 kJ K^{-1} mol^{-1} was observed (33). Larger values are observed when binding is coupled to folding and/or oligomerization; in the case of the λ Cro repressor, $\Delta C_p{}^o{}_{obs}$ is

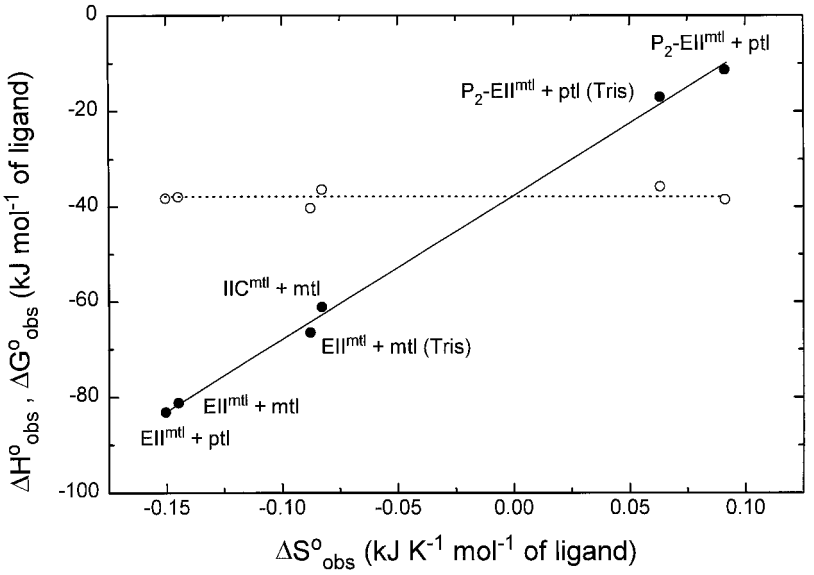

FIG. 7. **Enthalpy-entropy compensation for the binding of mannitol (*mtl*) and perseitol (*ptl*) to EII^{mtl}, P$_2$-EII^{mtl}, and C domain of enzyme II^{mtl} obtained from trypsinolysis (*IIC^{mtl}*), shown as a plot of ΔH^{o}_{obs} (●) and ΔG^{o}_{obs} (○) *versus* ΔS^{o}_{obs} at 25 °C (298 K).** Data were taken from Tables I–IV. The *solid line* is obtained from linear least squares analysis and has a slope of 303 ± 8 K. The *dotted line* represents the average value of ΔG^{o}_{obs} (−37.8 kJ mol).

−6.4 kJ K^{-1} mol^{-1} (34), and for the binding of the glucocorticoid DNA binding domain to its DNA site, $\Delta C_p{}^{o}_{obs}$ is −4.2 kJ K^{-1} mol^{-1} (35). Although EII^{mtl} is known to form dimers and, therefore, it could be argued that dimerization leads to the large value of $\Delta C_p{}^{o}_{obs}$ determined here, we do not believe that this is the case. The monomer-dimer equilibrium has been monitored by a number of techniques (3–9) and, in all of these cases, monomerization was observed only at concentrations in the nanomolar range and only in detergent. The concentrations we have employed in our study are much higher, and we are working with membrane vesicles with their natural complement of lipids. Thus, it seems fair to conclude that EII^{mtl} is in its dimeric form in all experiments in this study. Consequently, the observed thermodynamic parameters must result from changes in the EII^{mtl} dimer going from a free state to a substrate-bound state.

Spolar and Record (15) developed a method to determine the number of residues involved in a binding reaction from the value of $\Delta C_p{}^{o}_{obs}$. Application of their analysis to our data yields estimates of approximately 50–60 residues for the binding of mannitol and 62 for the binding of perseitol (Table V). This analysis depends on an average value of the ratio of polar and non-polar surface area that is buried during the binding process determined from data for globular proteins. Therefore, in the absence of structural data, it can only give an indication of the actual number of residues involved, but it illustrates that a major conformational change takes place upon binding of either mannitol or perseitol to the EII^{mtl}-dimer when the protein is embedded in the cytoplasmic membrane.

The Effect of Phosphorylation and Removal of the B Domain on $\Delta C_{p^{o}obs}$—In the case of perseitol binding to phosphorylated EII^{mtl}, $\Delta C_p{}^{o}_{obs}$ could not be determined accurately, but it is clear that the value is considerably smaller, although probably still rather large for the binding of a small molecule to a protein. The data do not permit a reliable calculation of the number of residues influenced by the binding, but we can qualitatively state that the number is much smaller when perseitol binds to phosphorylated *versus* unphosphorylated enzyme. Thus phosphorylation of the B domain influences the structural changes of the enzyme upon binding of perseitol to the C domain.

Removal of the A and B domain from EII^{mtl} by trypsinolysis decreases $\Delta C_p{}^{o}_{obs}$ to −0.5 kJ K^{-1} mol^{-1}. The theoretical value of the number of residue involved in the binding drops accordingly to a value of 9 residues, indicating that the conformational changes in the absence of the B and A domains involve considerably fewer residues than in the intact enzyme. Since it is known that there is no functional interaction between the A and C domains, we can attribute this difference solely to the absence of the B domain. Therefore, a very significant part of the structural changes occurring in the transporter upon binding of mannitol reside in the B domain, rather than the C domain.

Domain Interactions in EII^{mtl}—The results obtained after phosphorylation and trypsinolysis of the enzyme strongly suggest a conformational change upon binding of the substrate to the C domain that is propagated to the B domain. Other evidence for conformational coupling between the B and C domains of EII^{mtl} has been obtained from mannitol binding and transport kinetics and their dependence on chemical changes and mutations at the active site of the B domain (10, 11). Our data support those conclusions and also suggest a mechanism for the coupling. The very large value of $\Delta C_p{}^{o}_{obs}$ and the enthalpy-entropy compensation behavior indicate that a new complementary surface is formed upon binding, either from folding of unstructured parts or docking of preexisting surfaces on the protein, but, in either case, resulting in the removal of solvent-exposed parts of the protein from the surrounding water. This is most likely to happen between the B and C domains of the enzyme, because the next step in the catalytic cycle of the enzyme is phosphorylation of the incoming mannitol by the B domain, a process requiring precise positioning of the phosphoryl group donor and acceptor. This would explain the decrease in $\Delta C_p{}^{o}_{obs}$ after removal of the B domain because one half of the complementary surface is no longer present. The fact that phosphorylation of the protein also leads to a large decrease in $\Delta C_p{}^{o}_{obs}$ can be explained in two different ways; either the surface is already formed upon phosphorylation so that binding of substrate is no longer required to generate complementary surfaces, or the newly formed surfaces cannot complement due to steric repulsion of the C7 moiety of the perseitol and the phosphoryl group attached to Cys-384 on the B domain. A possible way to discriminate between the two possibilities is to study the thermodynamics of mannitol binding in a series of mutants that mimic the phosphorylated state of the B domain; these studies are in progress.

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